Annals of Clinical and Analytical Medicine
Original Research

In vitro characterisation of antioxidant and anti-inflammatory activities of ceratonia siliqua L. extracts and synthesised zinc oxide nanoparticles

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Abstract

Aim: This study aimed to investigate the phytochemical contents of extract of the Carob plant, which grows and is endemic in Cyprus, and to evaluate the antioxidant and anti-inflammatory activities of synthesised zinc oxide nanoparticles/Ceratonia siliqua L.

Material and Methods: Phytochemical analysis of Ceratonia siliqua L. extract was performed, and total flavonoid, phenolic and alkaloid contents were determined. Then, ZnO nanoparticles/Ceratonia siliqua L. were synthesised using zinc acetate and characterised using various techniques such as UV, FTIR, XRD and SEM. Hydroxyl radical, 2,2-diphenyl-1-picryl hydrazyl, and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) scavenging activities were used to determine as an antioxidant marker. To investigate the anti-inflammatory effect of Ceratonia siliqua L. extracts and ZnO-NPs/CsL, we performed assays such as heat-induced hemolysis, proteinase inhibitory activity and albumin denaturation tests.

Results: Our study results also revealed that Ceratonia siliqua L. extracts and synthesized ZnO-NPs showed a high radical capture potential and quite strong anti-inflammatory activity in vitro.

Discussion: Our study has shown that ZnO nanoparticles obtained by the green synthesis method have the potential to be used in a wide range of areas in medicine and will contribute to the literature in medical and technological terms.

Keywords

Antioxidant, Anti-Inflammatory, Ceratonia Siliqua L., Green Synthesis, ZnO Nanoparticles

DOI: 10.4328/ACAM.22109 Received: 2024-01-15 Accepted: 2024-02-24 Published Online: 2024-02-28 Printed: 2024-03-01 Ann Clin Anal Med 2024;15(3):210-215
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Introduction

Many scientific studies have extensively investigated the antioxidant and anti-inflammatory effects of compounds such as phenolic, terpenoid, and ketone. With the development of bio-compatible, bio-safe and environmentally friendly nanomaterials, nanotechnology will be widely used in medicine in the future [1-3]. Green biosynthesis of metal nanoparticles such as zinc oxide nanoparticles (ZnO-NPs) using different plant extracts is a new option without producing toxic chemicals [4]. Carob (Ceratonia siliqua L.) is native to the Mediterranean countries of Turkey, Greece, Portugal, Spain, Italy and Cyprus [5]. Free radicals are produced during normal physiological cell metabolism in the body. However, when they occur in large quantities, they disrupt the proper functioning of cellular functions, damage cell components, and cause chronic diseases [6, 7]. The urges taken in by humans through their diet and fruit contain antioxidant compounds. Ceratonia siliqua L. extract has been shown to contain high amounts of antioxidant phytochemicals, including phenols, tannins and flavonoids [6]. This herb is well known to have antioxidant activity for free radicals [8].

Inflammation is a complex biological response of body tissues to adverse stimuli such as infections, damaged cells or irritants. It is characterised by the production of cytokines involved in the regulation of inflammatory reactions. Atherosclerosis and cancer are associated with inflammatory diseases that have been linked to excessive chronic production of inflammatory cytokines. Numerous pieces of evidences have revealed that Ceratonia siliqua L. extracts exhibit anti-inflammatory effects by inhibiting myeloperoxidase activity, human neutrophil reactive oxygen species (ROS) production, and other inflammatory mediators [8].

Ceratonia siliqua L. has anti-inflammatory, anti-microbial and antioxidant properties [6,8], and the synthesis of nanoparticles of this plant has yet to be thoroughly studied. Therefore, this study aimed to evaluate the determination of the phytochemical contents (total flavonoid, phenolic and alkaloid contents) of the extract of Ceratonia siliqua L. and to characterise green synthesised ZnO nanoparticles/Ceratonia siliqua L. (ZnO-NPs/CsL) using various techniques such as UV, FTIR, XRD and SEM. Then, we searched the extract of Ceratonia siliqua L. and synthesised ZnO-NPs/CsL for their antioxidant and anti-inflammatory activities.

Material and Methods

Carobs were collected from the hills of Alsancak and Lapta in the Kyrenia district, Cyprus. Ceratonia siliqua L., examined in this study, was dried in the shade for two weeks, and the pulp and seeds of carobs were powdered with an electric plant grinder. All chemicals and zinc acetate dihydrate ($Zn(CH_3COO)_2.2H_2O$) to be used for the study were supplied by Merck Company (Germany).

Preparation of Ceratonia siliqua L. aqueous extract (CAE)

Firstly, 200 g of powdered Ceratonia siliqua L. was put into the balloon flask and boiled for 15 min at 80–90°C by adding 1 L of distilled water. Then, the mixture was cooled and filtered with a Whatman 1 filter paper. The extracts were stored at +4°C for research.

Phytochemical analysis of CAE

The total flavonoid content of CAE was determined using the aluminium chloride colorimetric method [9]. Initially, 0.2 mL of CAE was placed in a conical flask, 2 mL of distilled water and 750 µL of sodium nitrite solution was added, and incubated for 6 min at room temperature. Then 150 µL of $AlCl_3.6H_2O$ was added. Absorbance was measured at 510 nm using a Shimadzu UV 1800 Spectrophotometer (Japan). Total flavonoid content levels were made using a standard curve using the following equation based on the calibration curve: y=0.0049x+0.0047, R^2=0.9935, where y was the absorbance, and x was the quercetin. Quercetin was used as the reference material, and the calculation was done in mg of quercetin per g of sample.

The total phenolic content of CAE was determined using the Folin-Ciocalteu colorimetric method [9]. Briefly, 0.2 mL of CAE was mixed with 0.2 mL of Folin-Ciocalteau reagent and 2 mL of sodium carbonate solution. It was incubated at room temperature for two hours. The absorbance of the resulting blue colour solution was measured at 750 nm spectrophotometrically. Gallic acid was used as the reference material, and the calculation was made in mg of gallic acid per g of sample.

The total alkaloid content of CAE was determined according to the alkaloid-bromocresol green (BCG) reaction method, resulting in a yellow-coloured product [9]. 200 µL of CAE was placed in a test tube and 2 N HCl was added to make the final volume 1000 µL. The mixture was thoroughly mixed, transferred to a separatory funnel, and washed thrice using 10 mL of chloroform. The solution was neutralised by adding 0.1 N sodium hydroxide. After 10 min, 5 mL of BCG solution and 5 mL of phosphate buffer were added. The mixture was shaken, and the complex formed was extracted with chloroform. The extracts were collected and diluted with chloroform. Its absorbance was measured at 470 nm spectrophotometrically. The results were calculated using the formula [alkaloids= (Abs−0.048)/0.021] and expressed as mg-CAE/mL.

Synthesis and analysis of ZnO-NPs/CsL

To synthesise ZnO-NPs/CsL, 100 mL of CAE and 100 mL of zinc acetate solution were added to the beaker and kept at room temperature for one hour. The solutions were continuously heated and mixed at 100×g for four hours. The colour of the mixture changed from yellow to brown, indicative of ZnO-NPs/CsL formation. Next, it was purified by centrifugation with distilled water at 10000×g, 20 min. Supernatants were discarded, nanoparticle pellets were collected, washed with distilled water, freeze-dried using Alpha 1-2 LDplus and stored at -80°C [10]. The absorbance of ZnO-NPs/CsL was characterised at 200-800 nm, spectrophotometrically. The structural characterisation and particle size examination of ZnO-NPs/CsL were done by Transmission Electron Microscopy (TEM) and Scanning Electron Microscopic (SEM) (Hitachi, Japan). In addition, the spectra properties of those were carried out by Fourier Transform Infrared Spectroscopy (FTIR) (Bruker, Germany) in the spectra range of 4000–400 cm^{-1}. X-ray diffraction (XRD) of the ZnO-NPs/CsL was analysed using an XPert PRO diffractometer (Holland) in the 2θ range of 20–80° [10].

Antioxidant analysis of the ZnO-NPs/CsL

The determination of hydroxyl radical scavenging activity is

based on the degradation of 2-deoxy ribose by the hydroxyl radical produced by the Fenton system (Fe^{+3}, Ascorbic acid, EDTA, H_2O_2). The 2-deoxy ribose was condensed with thiobarbituric acid (TBA) and then measured as TBARS [9]. 45 µL of sodium phosphate buffer, 15 µL of deoxyribose, 150 µL of $FeSO_4$-EDTA, 15 µL of H_2O_2 and 525 µL of deionised water were added to 750 µL of CAE/or ZnO-NPs/CsL/or L-ascorbic acid (used as a control). The solution was kept in the incubator for four hours. 75 µL of trichloroacetic acid (TCA) and 75 µL of TBA were added, and the reaction was initiated by adding NaOH. Then, it was incubated in a water bath (90°C, 10 min), cooled, and centrifuged at 10000×g for 15 min. The supernatant was aspirated, and its absorbance was recorded at 520 nm spectrophotometrically. Percentage inhibition of hydroxyl radical was determined using the Eq-1: % Inhibition=[(Acontrol-Asample)/Acontrol]×100

To determine 2,2-diphenyl-1-picryl hydrazyl (DPPH) activity [11], from the stock solution, several doses of the sample (50-100-150-250-500 µg/mL) were prepared. 100 µL of DPPH solution was added to 100 µL of CAE/or ZnO-NPs/CsL/or L-ascorbic acid. The mixture was thoroughly vortexed and incubated in the dark (20°C, 30 min). The decrease in absorbance at 517 nm was measured spectrophotometrically to detect DPPH colour degradation. It was calculated using Eq-1, and from a concentration plot against %inhibition, a linear regression analysis was performed to determine the IC_{50} value (the sample concentration needed to remove 50% free radicals) [11].

The 2,2′-azino-bis (3-ethylbenzotiazolin-6-sulfonic acid)($ABTS^+$) test assesses the ability of antioxidants to clear the oxidative species produced by $ABTS^+$. $ABTS^+$ radicals are formed by a reaction between $ABTS^+$ and the highly oxidising potassium persulfate. As the antioxidant scavenging potential increased, a decrease in absorbance was observed [11]. ABTS was dissolved in water (7 mM). ABTS radicals were produced by adding potassium persulfate (2.45 mM). It was incubated in the dark (20°C, 12-16 h). 1 mL diluted of $ABTS^+$ solution was added to 10 µL of CAE/or ZnO-NPs/CsL/or L-ascorbic acid. After 6 min of incubation, the blue/green $ABTS^+$ solution turned pale yellow and then discoloured; absorbance was measured at 734 nm spectrophotometrically. Percentage inhibition of $ABTS^+$ was calculated using the Eq-1.

Anti-inflammatory analysis of the ZnO-NPs/CsL

In vitro, to investigate the anti-inflammatory effect of CAE or ZnO-NPs/CsL, we performed heat-induced haemolysis, proteinase inhibitory activity and albumin denaturation assays [12, 13]. Acetylsalicylic acid was used as a reference drug, and Eq-1 was used to calculate the %inhibition.

For erythrocyte suspension, whole blood was taken from a healthy person with a consent form by ethical rules. Blood with 0.9 % NaCl was centrifuged at 2000×g for 5 min and rinsed to remove the supernatant. The mixture was reconstituted as a 10% suspension by adding sodium phosphate buffer (10 mM/pH 7.4). For heat-induced haemolysis testing, 0.06 mL of CAE/or ZnO-NPs/CsL/or acetylsalicylic acid, 0.06 mL of blood cell suspension, and 2.93 mL of phosphate buffer were incubated in a water-bath (54°C), and then centrifuged again at 2000×g, 3 min; and its absorbance was measured at 540 nm spectrophotometrically.

In the proteinase inhibitor activity test, 0.06 mg of trypsin and 1 mL of Tris-HCl buffer (20 mM/pH 7.4) were added to 1 mL of CAE/or ZnO-NPs/CsL/or acetylsalicylic acid, and then incubated at 37°C/5 min. The mixture was incubated and centrifuged for another 20 min by adding 1 mL of casein (0.8%). 2 mL of 70% perchloric acid was added to the mixture, and its absorbance was measured at 210 nm spectrophotometrically.

To determine the inhibition of albumin denaturation, 0.2 mL of 1% bovine albumin and 3.8 mL of phosphate-buffered saline (pH 6.4) were added to 1 mL of CAE/or ZnO-NPs/CsL/or acetylsalicylic acid. It was incubated (at 37°C for 15 min and then at 70°C for 5 min). After cooling, its absorbance was measured at 660 nm spectrophotometrically.

Statistical Analysis

The data from antioxidant assays were subjected to descriptive statistics, expressed as x±SEM and analysed using One-Way ANOVA for statistical comparison of differences among means followed by Tukey's test for pairwise comparisons and values of $p<0.05$ were considered statistically significant.

Ethical Approval

All the reagents used in this study were prepared, used, and disposed of according to laboratory guidelines and material safety. Since no animals/humans were used during the study, the university ethics committee stated there was no need to get approval from the ethics committee.

Results

CAE's total flavonoid, phenolic and alkaloid contents were 289.26±2.5, 116.57±2.83, and 354.47±2.85, respectively. They were expressed as mg/100 g-CaE.

Our study used various techniques such as UV, FTIR, XRD, and SEM to control the synthesis of ZnO-NPs/CsL from CAE and characterise their catalytic activities. First, ZnO-NPs/CsL formation was analysed by UV spectroscopy. In general, the absorbance values of ZnO-NPs/CsL at 360 nm indicate that particles are synthesised [14]. The peak values in our study, 365 nm, supported ZnO-NPs/CsL synthesis (Figure 1A). The absorption peaks measured by FTIR spectroscopy refer to the tensile vibrations O-H (alkene), C=C, N-O, and C=O (carbonyl) bindings (Figure 1B), respectively. Metal oxides usually give vibration frequencies below 1000 cm^{-1}, and their peaks in the spectrum between 470 and 600 cm^{-1} are indicated to belong to ZnO-NPs/CsL.

Zeta potential testing was used to assess the electrical charge on the surface of ZnO-NPs, and XRD analysis was used to reveal the crystal structure of the synthesised ZnO-NPs/CsL (Figure 2A). As shown in the figure, the peaks were shown with 2θ values of 35.46, 37.01, 43.52, 48.61, 52.39, 64.26, and 75.44. These peaks corresponded to (100), (002), (101), (102), (110), (103), and (200), respectively. Finally, the SEM images revealed particles with rounded morphologies and an average size of 200 µm (Figure 2B). The results determined for the hydroxyl radical and DPPH scavenging or $ABTS^+$ activities of CAE and ZnO-NPs/CsL are presented in Table 1. It was observed that the maximum antioxidant activities were at 500 µg/mL. The %inhibitions of the hydroxyl radical, DPPH and $ABTS^+$ scavenging of the CAE were 47.82±0.03%, 54.68±0.12%, and 65.58±0.02%, respectively. These inhibitions

for ZnO-NPs/CsL were 41.17±0.06%, 44.52±0.21%, and 49.58±0.04%, respectively. They were determined for the standard L-ascorbic acid as 49.26±0.08%, 58.21±0.08%, and 69.42±0.05%, respectively. The %inhibition values for CAE and ZnO-NPs/CsL were significantly lower than that of standard L-ascorbic acid, indicating excellent free radical inhibition at lower concentrations. We performed heat-induced haemolysis to determine the anti-inflammatory effects of CAE and ZnO-NPs/CsL. We found that the IC_{50} values for acetylsalicylic acid (2.06±0.84 mg/mL), CAE (3.89±0.72 mg/mL), and ZnO-NPs/CsL (3.08±0.97 mg/mL). In the proteinase inhibitory activity test, we determined IC_{50} values for acetylsalicylic acid (2.21±0.57 mg/mL), CAE (3.57±0.31 mg/mL), and ZnO-NPs/CsL (2.96±0.82 mg/mL). When we performed the albumin denaturation inhibition, the IC_{50} values were found for acetylsalicylic acid, CAE and ZnO-NPs/CsL as 3.01±0.63 mg/mL, 4.51±1.21 mg/mL, and 3.67±1.05 mg/mL, respectively. The values were increased with the decrease in the concentration of our measured test agents (Table 2).

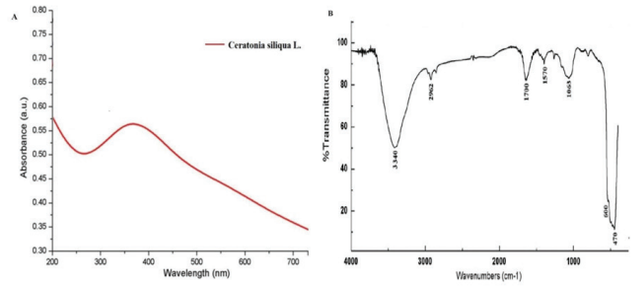

Figure 1. UV spectra of ZnO-NPs/CsL, and B: FTIR spectra of synthesized ZnO-NPs/CsL by Ceratonia siliqua L.

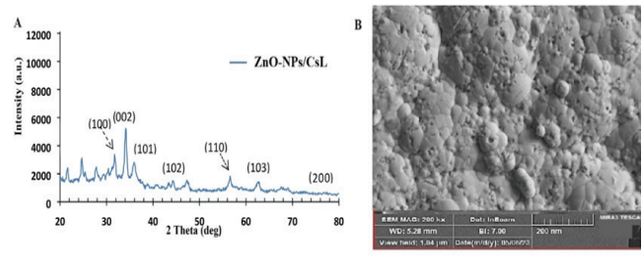

Figure 2. XRD spectra of synthesized zinc oxide nanoparticles using Ceratonia siliqua L., and B: SEM images of synthesized ZnO-NPs/CsL using Ceratonia siliqua L.

Table 1. Hydroxyl, DPPH, and ABTS+ radical scavenging activities of CAE and ZnO-NPs/CsL, in vitro*

Concentration (µg/ml)	%inhibition ± SEM		
	%Hydroxyl inhibition of CAE	%Hydroxyl inhibition of ZnO-NPs/CsL	Standard (L-Ascorbic acid)
50	41.12±0.06^{Ca}	36.94±0.07^{cA}	43.57±0.08^{bc}
100	42.50±0.05B^{Ca}	38.57±0.09^{bc}	45.98±0.08^{bC}
150	44.45±0.03^{BA}	40.21±0.08^{ABc}	46.33±0.08^{aA}
250	46.94±0.04^{Aa}	41.02±0.40^{AB}	48.14±0.08^{bA}
500	47.82±0.03^{cA}	41.17±0.06^{CA}	49.26±0.08^{bA}
IC_{50}(mg/mL)	10.420	21.570	33.200
	%DPPH inhibition of CAE	%DPPH inhibition of ZnO-NPs/CsL	Standard (L-Ascorbic acid)
50	49.86±0.04^{bC}	38.75±0.03^{AB}	53.42±0.07^{bc}
100	51.47±0.03^{CA}	39.86±0.08^{bc}	55.38±0.05^{Ca}
150	52.24±0.11^{ABc}	41.58±0.03^{BA}	56.51±0.03^{aA}
250	55.92±0.22^{cA}	42.38±0.05^{bA}	57.55±0.24^{cA}
500	54.68±0.12^{Aa}	44.52±0.21^{BCa}	58.21±0.08^{bA}
IC_{50}(mg/mL)	15.380	18.750	28.920
	%ABTS+ inhibition of CAE	%ABTS+ inhibition of ZnO-NPs/CsL	Standard (L-Ascorbic acid)
50	61.22±0.11^{bC}	46.03±0.05^{Ac}	63.32±0.05^{aA}
100	62.27±0.08^{BC}	46.52±0.14^{Bc}	65.58±0.15^{bA}
150	63.45±0.07^{cA}	47.18±0.05^{AB}	66.30±0.02^{bc}
250	64.41±0.03^{cb}	48.78±0.03^{Ab}	68.35±0.07^{bA}
500	65.58±0.09^{cB}	49.58±0.04^{CA}	69.42±0.0^{bC}
IC_{50}(mg/mL)	15.600	19.320	28.400

*Values were expressed as x±SEM. Mean values (x) were taken after three parallel measurements. Values with the same lowercase superscript letter across the rows and uppercase superscript letter along the columns were not significantly different (p>0.05; one-way ANOVA followed by Tukey's test).

Table 2. The anti-inflammatory effects of CAE and ZnO-NPs/CsL*

Test agents	Concentration of the extract (µg/ml)	Heat-induced haemolysis (IC_{50} mg/mL)	% Proteinase inhibitory activity (IC_{50} mg/mL)	Albumin denaturation (IC_{50} mg/mL)
CAE	50, 100, 150, 250, 500	3.89±0.72	3.57±0.31	4.51±1.21
ZnO-NPs/CsL	50, 100, 150, 250, 500	3.08±0.97	2.96±0.82	3.67±1.05
Acetylsalicylic acid	50, 100, 150, 250, 500	2.06±0.84	2.21±0.57	3.01±0.63

*Acetylsalicylic acid was used as the reference drug for the experiment. The anti-inflammatory effect of test agents in the study increased with the decrease in their concentration.

Discussion

We determined the phytochemical contents of CAE in our study. The phenolic compounds have been discovered to be the most abundant plant substances with antioxidant activity. The flavonoids also are thought to have an antioxidant effect through a sweeping or chelating process. Previous studies of the Carob plant have shown that it contains significant amounts of phenolics and flavonoids, while Carob also contains high amounts of alkaloids [1]. There are also many reports indicating the presence of alkaloids in Carob [15], which may explain its traditional therapeutic uses. Many studies have shown that these molecules are well-known for their antioxidant and anticancer properties [14].

In our study, ZnO-NPs/CsL were synthesised using zinc acetate as a precursor. They characterised their catalytic activities using techniques such as UV, FTIR, XRD and SEM. First, ZnO-NPs/CsL formation was analysed by UV spectroscopy. In general, the absorbance values of ZnO-NPs/CsL at 360 nm indicate that particles are synthesised [14]. The peak values in our study, 365 nm, supported ZnO-NPs/CsL synthesis. The absorption peaks measured by FTIR spectroscopy refer to the tensile vibrations O-H (alkene), C=C, N-O, and C=O (carbonyl) bindings, respectively. CAE contained the phenolic compound, allowing the detection of the presence of phytochemicals and functional groups by FTIR analysis ranging from 400-4000 nm [14]. Metal oxides usually give vibration frequencies below 1000 cm^{-1}, and their peaks in the spectrum between 470 and 600 cm^{-1} are indicated to belong to ZnO-NPs/CsL. The peak recorded at about 1700 cm^{-1} in our study implies alkene (C=C) and carbonyl (C=O) stretching, while the peak recorded at 1570 cm^{-1} was assigned to the aromatic bending vibration of the alkane group (C-H). Zeta potential testing was used to assess the electrical charge on the surface of ZnO-NPs, and XRD analysis was used to reveal the crystal structure of the synthesised ZnO-NPs/CsL. When the obtained XRD peaks were examined, they were consistent with the literature data [16].

ZnO-NPs are unique molecules due to their oxidation state. They are positively charged on their surfaces due to the gap in their "d and f " orbitals and show antioxidant properties by reducing and scavenging electrons from other molecules. ROS are generated in the body during normal cellular metabolism. ROS are highly reactive and cause rapid oxidation of target molecules that lead to lipid peroxidation in cells among many biochemical reactions [17], thus causing many diseases caused by oxidative stress such as neurological diseases, old age, cancer and heart disease [18, 11]. ZnO-NPs effectively bind free radical species, hydroxyl radical, hydrogen peroxide, superoxide anion radical, and singlet oxygen, reducing oxidative stress. Medicinal plants contain high amounts of antioxidant phytochemicals, including phenols, tannins and flavonoids, and medicinal plants have been observed to prevent lipid peroxidation due to synthesis with ZnO [19, 20]. The determinations of hydroxyl radical, DPPH, and ABTS scavenging activities have long been used as an antioxidant marker in studies related to oxidative stress and focused on investigating the antioxidant effect of plants. Plant extracts whose hydroxyl radical, DPPH and $ABTS^{+}$ radical scavenging activities were determined to have remarkable antioxidant potential [21]. Darwish et al. [22] reported that CAE exhibited potent antioxidant activity that prevented the formation of DPPH, hydroxyl, and nitric oxide free radicals. They suggested that the scavenging activity of the CAE was often evaluated according to its IC_{50}; the lowest IC_{50} value corresponds to the highest free radical scavenging activity. We suggested that the studied CAE and ZnO-NPs/CsL are potent scavengers of ROS radicals in vitro and, therefore, have high antioxidant activity, as evidenced by low IC_{50} values.

In vitro, to investigate the anti-inflammatory effect of CAE and ZnO-NPs/CsL, we performed heat-induced haemolysis, proteinase inhibitory activity and albumin denaturation assays. For the potential anti-inflammatory effect of the medicinal plant Ceratonia siliqua L., it is used in various diseases, including diabetes, especially in Anatolia (Turkey) and Cyprus. It exerts its anti-inflammatory effects in controlling protein denaturation and albumin denaturation by inhibiting enzymes such as serine proteinase, which is released from lysosomal granules of neutrophils and has documented that leukocyte proteinase plays an essential role in the development of tissue damage during inflammatory disorders such as rheumatoid arthritis and diabetes. The literature has also shown that CAE has anti-inflammatory potential because it contains phenolic compounds, which are used for their anti-inflammatory and analgesic effects, strengthening the immune system, relieving pain, and treating skin disorders and asthma [23-25].

Conclusion

Our study indicated that CAE contained phenolic compounds and possessed antioxidant activity. ZnO-NPs/CsL have been shown to have antioxidant effects by reducing biomolecular agents in the structure of the plant with radical quenching activity. They also have been found to have a potent anti-inflammatory agent that protects cells against inflammation. Therefore, it is thought that ZnO-NPs/CsL, whose production and consumption potential is increasing, can provide many advantages to humanity in the treatment of oxidative/inflammatory diseases and cancer. Therefore, further investigations need to be conducted to investigate the effect of CAE and ZnO-NPs/CsL.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and Human Rights Statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or compareable ethical standards.

Funding: *None*

Conflict of Interest

The authors declare that there is no conflict of interest.

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How to cite this article:
Emrah Caylak, Gokhan Nur. *In vitro characterisation of antioxidant and anti-inflammatory activities of ceratonia siliqua L. extracts and synthesised zinc oxide nanoparticles. Ann Clin Anal Med 2024;15(3):210-215*